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Blockade of neuromuscular transmission was produced in the lower hind limb of the rat by local injection of either type A or type F botulinum toxin (BoTx). At 1, 3, 7 and 10 days after injection, the extensor digitorum longus (edl) nerve-muscle preparation was excised and analyzed for alterations in muscle mechanical properties or spontaneous and nerve stimulus-evoked quantal transmitter release. Muscles receiving type A toxin were paralyzed up to and including 7 days after injection. Muscles treated with type F toxin, although completely paralyzed at 1 and 3 days, twitched in response to nerve stimulation			

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by 7 days after injection. Both toxins induced a marked decrease in the frequency of miniature endplate potentials, but type A did so to a great extent. At 1 and 3 days after toxin injection nerve impulse evoked transmitter release was reduced for both type A- and type F-treated muscles. However, 3,4-diaminopyridine (3,4-DAP), an agent which increases nerve-evoked transmitter release by increasing  $Ca^{2+}$  influx, was more effective in reversing the paralysis in type A- than in type F-treated muscles. 3,4-DAP induced asynchronous end-plate potentials in response to nerve stimulation in type F-paralyzed muscles, but not in muscles treated with type A. Amidation of amino groups (presumably lysine) by treatment of type A toxin with ethylacetimidate had no effect on toxicity in mice or duration of paralysis of the edl in rats. Amidation of type F both increased toxicity and duration of paralysis. The results show that type F BoTx differs from type A, mainly by its lower potency, shorter duration of action, and by being less effectively antagonized by 3,4-DAP.



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COMPARISON OF THE ACTION OF TYPES A AND F  
BOTULINUM TOXIN AT THE RAT NEUROMUSCULAR JUNCTION

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In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The view of the author(s) do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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## ABSTRACT

Comparison of the action of types A and F botulinum toxin at the rat neuromuscular junction. Kauffman, J. A., Way J. F., Jr., Siegel, L. S. and Sellin, L. C. (1984). Toxicol. Appl. Pharmacol. . . . Blockade of neuromuscular transmission was produced in the lower hind limb of the rat by local injection of either type A or type F botulinum toxin (BoTx). At 1, 3, 7 and 10 days after injection, the extensor digitorum longus (edl) nerve-muscle preparation was excised and analyzed for alterations in muscle mechanical properties or spontaneous and nerve stimulus-evoked quantal transmitter release. Muscles receiving type A toxin were paralyzed up to and including 7 days after injection. Muscles treated with type F toxin, although completely paralyzed at 1 and 3 days, twitched in response to nerve stimulation by 7 days after injection. Both toxins induced a marked decrease in the frequency of miniature endplate potentials, but type A did so to a greater extent. At 1 and 3 days after toxin injection nerve impulse evoked transmitter release was reduced for both type A-and type F-treated muscles. However, 3,4-diaminopyridine (3,4-DAP), an agent which increases nerve-evoked transmitter release by increasing  $Ca^{2+}$  influx, was more effective in reversing the paralysis in type A-than in type F-treated muscles. 3,4-DAP induced asynchronous end-plate potentials in response to nerve stimulation in type F-paralyzed muscles, but not in muscles treated with type A. Amidination of amino groups (presumably lysine) by treatment of type A toxin with ethylacetimidate had no effect on toxicity in mice or duration of paralysis of the edl in rats. Amidination of type F both increased toxicity and duration of paralysis. The results show that type F BoTx differs from type A, mainly by its lower potency, shorter duration of action, and by being less effectively antagonized by 3,4-DAP.

## INTRODUCTION

The neurotoxins of Clostridium botulinum are found as at least seven immunologically distinct types A-G (Sugiyama, 1980). This antigenic distinction suggests probable differences in the structure-function relationship among these toxin types. Although all botulinum toxins (BoTx) are capable of blocking transmitter release at the neuromuscular junction, interesting qualitative and quantitative differences have been observed when comparing the effects of the various types (Harris and Miledi, 1971; Sellin et al., 1983a; Sellin et al., 1983b).

Purified type A BoTx was shown to be about 10x more lethal per unit protein than type F after ip injection in mice (Yang and Sugiyama, 1975; Das Gupta and Sugiyama, 1977a, 1977b). Therefore, we have compared the effects of BoTx types A and F at neuromuscular transmission in the rat. Since 3,4-diaminopyridine (3,4-DAP) antagonized the paralysis caused by type A BoTx (Molgo, et al., 1980) and is therefore a potentially useful compound for treatment of botulism, we also examined its effect on the neuromuscular block produced by type F.

In order to determine the basis for the lower potency of type F BoTx, chemical modification of the protein toxins was done by amidination of amino groups (Hunter and Ludwig, 1972). A previous study demonstrated increases in ribonuclease activity after treatment of the enzyme with a bifunctional imidoester (Hartman and Wold, 1967). In a similar fashion we treated BoTx type A and F with the imidoester ethylacetimidate in an effort to ascertain what factors may be critical to their toxicities.

## METHODS

Clostridium botulinum type A strain Hall was cultivated in a fermenter system (Siegel and Metzger, 1979). The method used for purification of the toxin was a modification of those described previously (Duff et al., 1957; DasGupta and Boroff, 1967; Sugiyama, et al., 1977). The toxin was precipitated from the culture fluid by adjusting the pH to 3.5, extracted with 0.2 M phosphate buffer, pH 6.0, dialyzed against 0.05 M citrate buffer, pH 5.5, and applied to a DEAE cellulose column equilibrated with citrate buffer. Toxin emerged in the void volume and fractions having a 260/280 nm absorbance ratio of 0.50-0.56 were pooled. After concentration with polyethyleneglycol, the toxin was dialyzed against 0.05 M phosphate buffer, pH 7.9 and applied to a DEAE cellulose column equilibrated in the phosphate buffer. Upon elution with phosphate buffer, the neurotoxin emerged in the void volume. The neurotoxin was then dialyzed against 0.2 M succinate buffer, pH 5.5. This preparation was approximately 85% pure, (as determined by SDS polyacrylamide gel electrophoresis) and was free of detectable hemagglutinin activity.

The Langeland strain of type F was cultivated as previously described (Yang and Sugiyama, 1975). The toxin was precipitated from the culture fluid by adjusting the pH to 4.0, extracted with 0.2M phosphate pH 6.0 and reprecipitated with ammonium sulfate (Oishi and Sakaguchi, 1974). The precipitated toxin was dissolved in 0.07 M phosphate pH 6.0, dialyzed against that buffer, and applied to a DEAE cellulose column equilibrated in the phosphate (Yang and Sugiyama, 1975). After washing the column with the phosphate buffer, the toxin was eluted with the same buffer containing 0.15 M NaCl (Yang and Sugiyama, 1975). This preparation was then dialyzed against 0.2M succinate pH 5.5. and was determined to be approximately 80% pure.

Toxicity was measured by mouse bioassay as previously described (Siegel and Metzger, 1979). All doses of toxin mentioned in this study are expressed as mouse ip LD<sub>50</sub>. In some experiments concentrated solutions ( $2 \times 10^5$  LD<sub>50</sub>/ml) of type A or F BoTx were treated with 0.2 M ethylacetimidate (Sigma) in a 28 mM Na<sub>2</sub>HPO<sub>4</sub> buffer solution for 30-40 min. These solutions were then diluted by at least 4000x before injection into mice or rats. Injection of appropriate concentrations of ethylacetimidate were used as controls.

Experiments were performed in situ or in vitro on the extensor digitorum longus (edl) muscle of male Wistar rats (100 to 200 g). A single 0.25 ml bolus of various doses (2-2000 LD<sub>50</sub>) of either type A or type F BoTx was injected subcutaneously into the anterolateral region of the right hind leg, superficial to the distal part of the tibialis anterior muscle. At 1, 3, 7 or 10 days after injection, the edl nerve-muscle preparation was examined for alterations in muscle mechanical properties (in situ) or electrophysiological properties (in vitro), using techniques described previously (Sellin et al., 1983a; Sellin et al., 1983b).

## RESULTS

The ip LD<sub>50</sub> for the rat was estimated to be 25 and 500 mouse ip LD<sub>50</sub> for BoTx types A and F, respectively. The rat sc LD<sub>50</sub> was about 60 mouse ip LD<sub>50</sub> for type A and 8500 mouse ip LD<sub>50</sub> for type F.

Partial or complete paralysis of one lower hind limb was observed between 12 and 24 hours. Type A BoTx was more potent and more persistent in its paralytic action than type F (Fig. 1). For example, muscles treated with a type A dose of 50 LD<sub>50</sub> were paralyzed up to 7 days after toxin injection, and single twitch tension remained less than 25% of normal at 10 days after injection. In contrast, muscles receiving 200-2000 LD<sub>50</sub> of type F were

paralyzed at 1 and 3 days, but single twitch tension was normal or greater than normal at 7 and 10 days after toxin injection. Although not shown, values of tetanic tension (nerve stimulation at 80 Hz) gave a qualitatively similar pattern of paralysis and recovery as illustrated in Fig. 1.

The normal (untreated) edl had a miniature end-plate potential (m.e.p.p.) frequency of  $2.04 \pm 0.07 \text{ s}^{-1}$ . Types A and F BoTx reduced m.e.p.p. frequency recorded from edl muscles as shown in Fig. 2. At 1 day after injection of type A doses of 2 and 20 LD<sub>50</sub>, m.e.p.p. frequency decreased to less than 1/200 of its normal value. By 10 days after toxin injection m.e.p.p. frequency had increased only slightly. Doses of type F between 2-2000 LD<sub>50</sub> also reduced m.e.p.p. frequency, but to a lesser extent. In contrast to type A, the m.e.p.p. frequency recorded from type F-treated muscles increased substantially over time, reaching normal or above normal values at 7 and 10 days after toxin injection.

End-plate potentials (e.p.p.s.) were analyzed between 1 and 3 days after injection of either type A or F BoTx. The effect of temperature on mean quantal content ( $\bar{m}$ ) for type A-or F-treated muscles is shown in Fig. 3. In type A-treated preparations  $\bar{m}$  increased significantly as the temperature was lowered from 30 to 20°C. In contrast, muscles treated with type F toxin showed a slight decrease in  $\bar{m}$  with the same decrease in temperature.

The addition of 3,4-DAP, a substance known to increase transmitter release in muscles poisoned with BoTx type A (Molgo et al., 1980, Sellin, et al., 1983), increased the amplitude and frequency of e.p.p.s. at 1 Hz nerve stimulation for both type A-and type F-treated muscles, but did so with different efficacies (Fig. 4). That is, 3,4-DAP was less effective in reversing the blockade in muscles treated with type F BoTx than those treated with type A. Addition of 100  $\mu\text{M}$  3,4-DAP caused all type A-treated muscles to



twitch after nerve stimulation. None of the muscles treated with type F toxin twitched after nerve stimulation in the presence of 100  $\mu$ M 3,4-DAP although the frequency and amplitude of e.p.p.s. increased. In addition, high concentrations of 3,4-DAP, produced asynchronous release in type F-treated muscles, but not in those receiving type A (Fig. 5).

Treatment with ethylacetimidate consistently increased the toxicity of type F BoTx by 1.5 - 8X after ip injection in mice. Modified type F BoTx was also more persistent in its paralytic action than native type F BoTx in paired experiments (Fig. 6). The latter observation was unrelated to mere increases in dose levels (see Fig. 1) and did not inhibit neutralization of the toxin by specific antibody. These alterations in activity were not observed in muscles treated with type A BoTx modified with ethylacetimidate.

#### DISCUSSION

The results demonstrate interesting quantitative and qualitative differences in the blockade of transmitter release produced by BoTx types A and F at the rat neuromuscular junction. Both toxins were capable of producing muscle paralysis, but type A was more potent and persistent in its paralytic action than type F. This fundamental difference in potency and persistence of action was also demonstrated in the toxins' effect on spontaneous quantal release, as measured by the frequency of miniature end-plate potentials. These observations suggest that structural differences between types A and F BoTx may cause F to interact less effectively with its binding site, the site responsible for neuromuscular blockade, and/or to undergo detoxification more rapidly than type A.

The experiments with ethylacetimidate-modified BoTx suggest one basis for the lower potency and shorter duration of action of type F BoTx. The reaction

of imidoesters with protein amino groups forms amidines which are stronger bases than their parent amines (Hunter and Ludwig, 1972). This reaction is presumed to occur at lysine residues and, therefore, these moieties may play an important role in determining the general toxicity and persistence of type F BoTx.

Both type A and type F BoTx reduced nerve impulse-evoked transmitter release. However, the blockade produced by these two toxin types differed in at least two important respects, sensitivity to temperature and sensitivity to 3,4-DAP. A recent study (Lundh, 1983) demonstrated that decreasing the temperature in vitro restores muscle twitch after nerve stimulation in a muscle previously paralyzed by BoTx type A. The present data qualitatively confirm this observation for type A-treated muscles. However, muscles treated with type F BoTx showed little or no change in quantal content with a 10°C change in temperature. In addition, 3,4-DAP, which greatly increased nerve-impulse-evoked  $Ca^{++}$  influx and thereby transmitter release, was more effective in restoring neuromuscular transmission in type A-than type F-treated preparations. Instead, nerve stimulation in the presence of 3,4-DAP caused asynchronous release in type F-treated muscles. A similar form of asynchronous transmitter release was observed in nerve terminals treated with type B BoTx (Sellin et al., 1983b), type D BoTx (Harris and Miledi, 1971) and tetanus toxin (Dreyer and Schmitt, 1981).

The specific site and mode of action by which BoTx inhibits transmitter release is not known. Final elucidation of the toxic mechanism may remain obscure until we better understand the normal process of transmitter release. However, one may speculate regarding those systems which have been implicated as factors in the vesicular release of neurotransmitter and, therefore, may be targets for BoTx. Synapsin I is a collective name for two

similar proteins associated with synaptic vesicles in nerve endings (De Camilli et al., 1983). Synapsin I is a major endogenous substrate for cAMP-dependent phosphorylation and  $\text{Ca}^{++}$ /calmodulin-dependent protein kinases in mammalian brain. Since transmitter release itself can be regulated both by  $\text{Ca}^{++}$  and cAMP, Synapsin I, the cAMP-dependent phosphorylation system, or the  $\text{Ca}^{++}$ /calmodulin-protein kinases may provide a substrate for BoTx. The mechanisms for the mobilization (e.g. phosphatidylinositol turnover, [Prentki et al., 1984] and sequestration of  $\text{Ca}^{++}$  (Thesleff, personal communication) may also be affected by BoTx.

In conclusion, given the differences in the binding (Williams et al., 1983) and the pharmacological actions (Sellin et al., 1983a; Sellin et al., 1983b; Harris and Miledi, 1971) of the various BoTx types, one should not make generalizations concerning all types of BoTx based on the actions of a single type. The various types may act differently during binding to the extracellular face of the nerve terminal, internalization of the toxin and/or in the final paralytic step.

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Fig. 1. Single twitch tension, normalized for differences in weight, for types A and F BoTx plotted against days after sc injections. Normal single twitch tension in unpoisoned muscles is also illustrated. Each point is the mean  $\pm$  S.E. for at least 10 muscles.

Fig. 2. M.e.p.p. frequency recorded from edl muscles treated with various doses of type A or F BoTx plotted against days after sc injection. Normal M.e.p.p. frequency for unpoisoned muscles is also given. Points represent mean  $\pm$  S.E. for at least 100 fibers in 10 muscles.

Fig. 3. Mean quantal content ( $\bar{m}$ ) e.p.p. recorded from edl muscles treated with various doses of type A or F BoTx plotted against change in temperature. Values are mean  $\pm$  S.E. of 10 fibers in 2 muscles for BoTx and 10-15 fibers in 3 muscles for BoTx F.

Fig. 4. Effect of 3,4-DAP on the neuromuscular blockade produced by various doses of types A or F BoTx at 1 and 3 days after poisoning. The graph shows the number of nerve stimuli causing transmitter release (e.p.p.) as a percentage of the total number of stimuli at 1 Hz. Each point represents the mean  $\pm$  S.E. for at least 100 fibers in 10 muscles. Note that at both time points 100  $\mu$ M 3,4,-DAP BoTx A-poisoned muscle twitched, while increasing transmitter release only somewhat in type F-treated preparations.

Fig. 5. Examples of e.p.p. (superimposed tracings) in type A- and F-poisoned muscles 3 days after injection and the effects of 10 and 100  $\mu$ M 3,4-DAP. Stimulation frequency was 1 Hz. Note that in type A-poisoned muscles, 3,4-DAP enhanced synchronous transmitter release and at 100  $\mu$ M caused the muscle to twitch (lowest record). In contrast, 3,4-DAP caused asynchronous transmitter release and no twitches in a type F-poisoned muscle.

Fig. 6. Single twitch tension for 1600 LD<sub>50</sub> F BoTx and modified 1600 LD<sub>50</sub> F BoTx (modified with ethylacetimidate) plotted against days after sc injection. Each point is the mean  $\pm$  S.E. for 3 muscles of F BoTx and 4 muscles of modified F BoTx. Note the difference in duration of paralysis.



FIG 1

SINGLE TWITCH TENSION

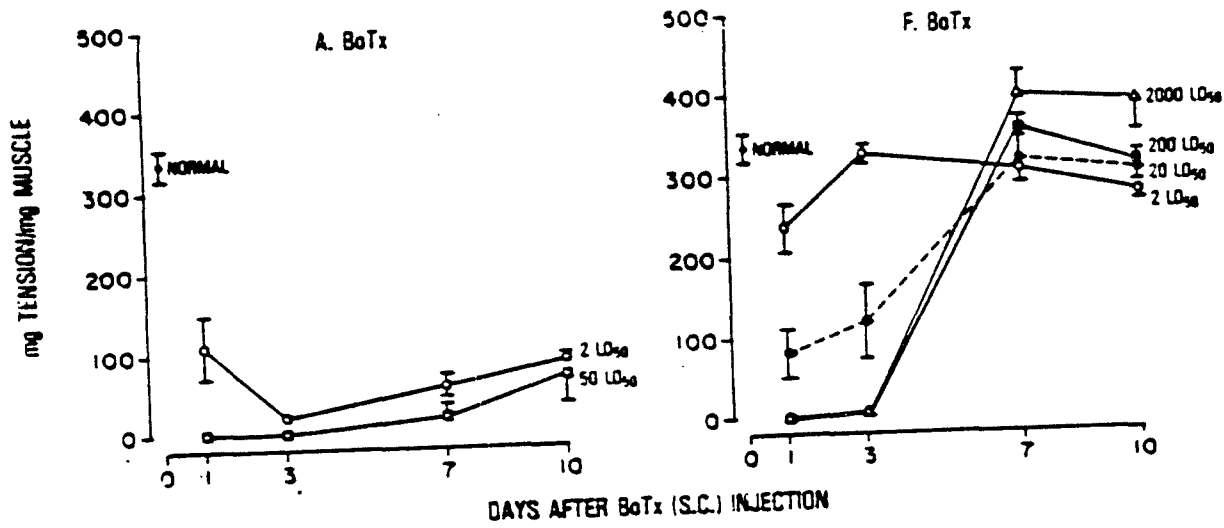


FIG 2

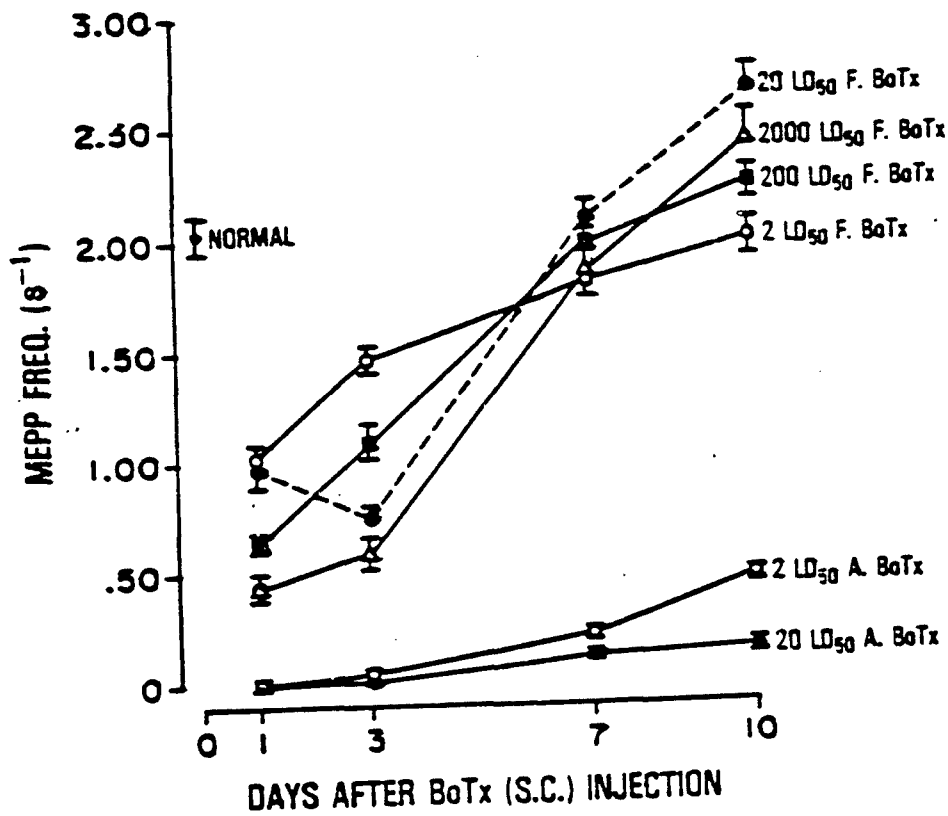


FIG 5

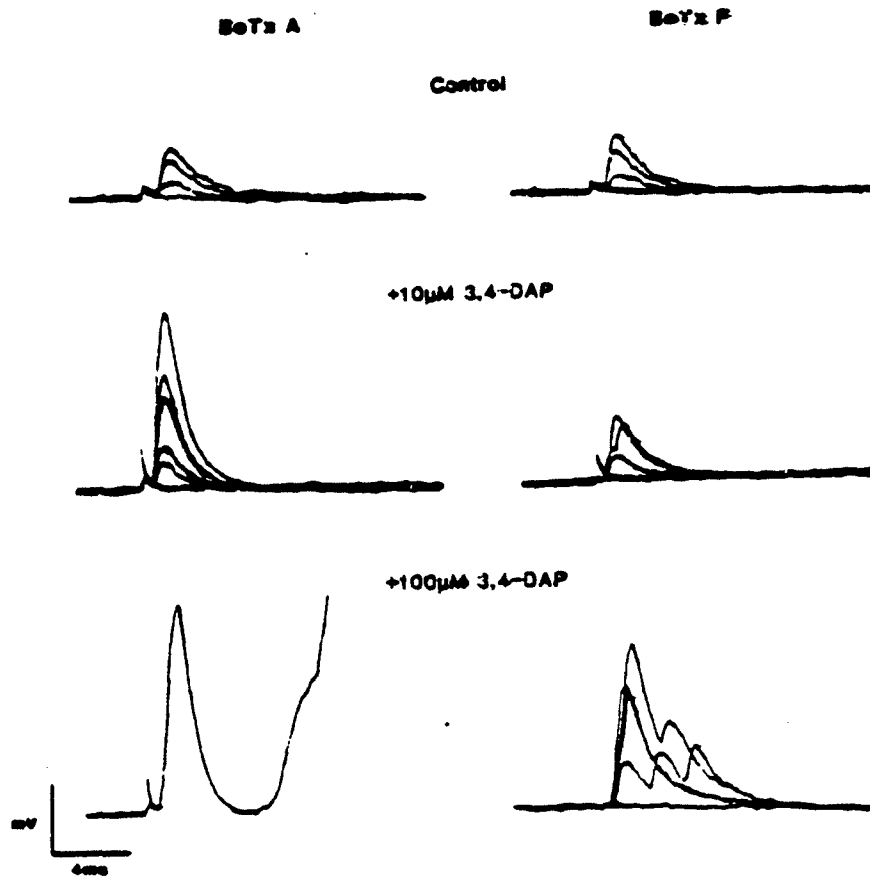


FIG 6

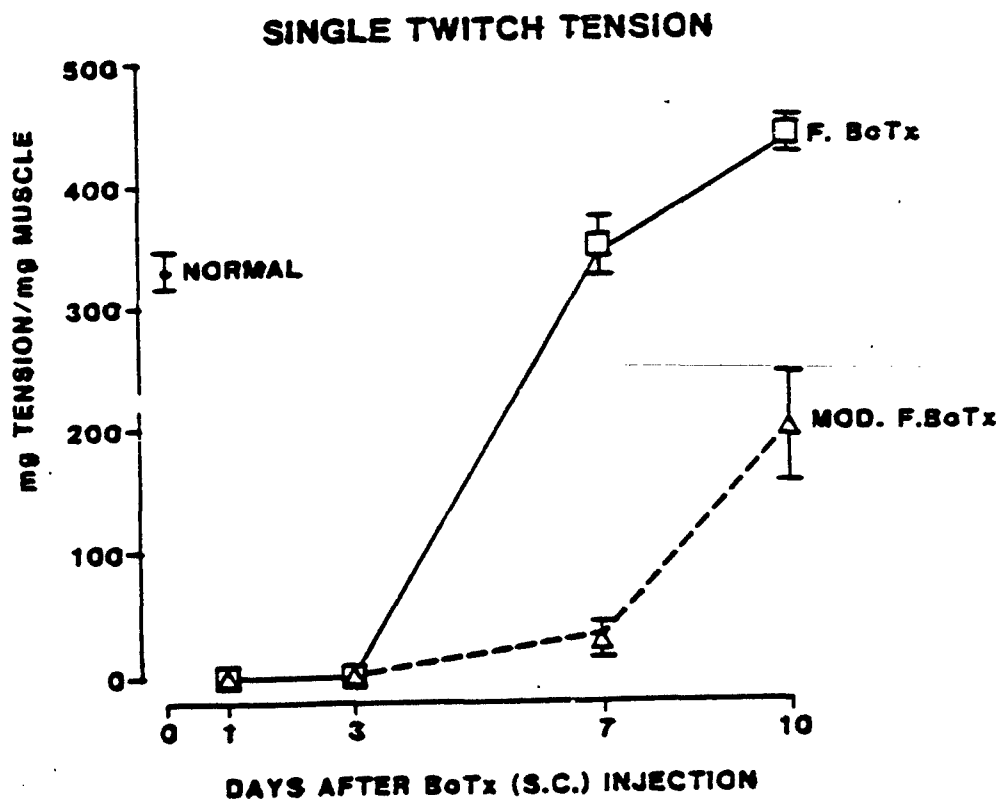


FIG 3

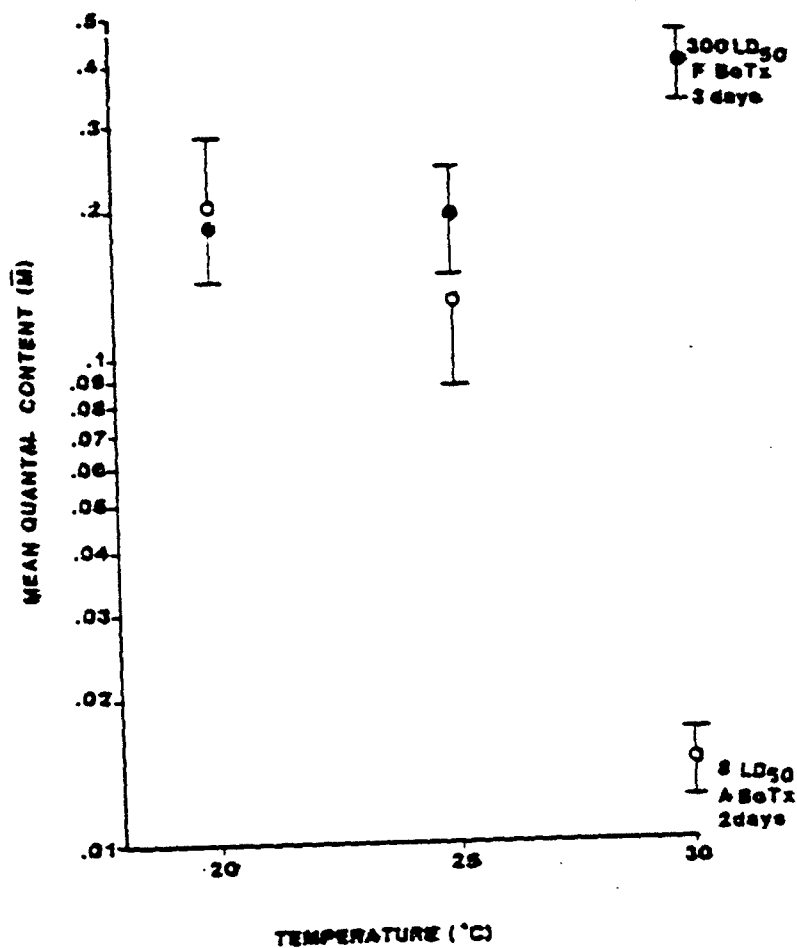


FIG 4

